DESCRIPTION

NOVEL GLUTAMIC ACID RECEPTOR AND UTILIZATION THEREOF

Technical Field

The present invention relates to novel glutamic acid receptor and utilization thereof, and more specifically to a glutamic acid receptor, DNA which encodes the receptor, a method of screening a ligand that binds to the receptor utilizing the same, a receptor binding test and so forth.

Background Art

Glutamic acid is the major excitatory neurotransmitter in the central nervous system and it is widely accepted that anomaly of its control is involved in creation of pathological conditions of progressive encephalopathies such as disorder in memory, ischemic encephalopathy, amyotropic lateral sclerosis (ALS), Parkinson's disease, and Huntingon's chorea (Meldrum, B.S., Meruolgy, 1994 Nov;44 (11 Supple 8):S14-23; Nishizawa, Y., Life Sci. 2001, Jun 15;69(4):369-81). This led to many studies on glutamic acid receptors throughout the cranial nerves, resulting in discovery of many types of receptors (three ionic type receptors and eight metabolic type receptors) in the central nervous system. With a view to developing therapeutic medicines for the above-mentioned diseases, even now development of agonists specific to such receptors is being energetically made (for details, reference is made to Barnard, E.A., Trends Pharmacol. Sci., 1997, May; 18(5):141-8; Schoepp, D.D., Conn, P.J., Trends

Pharmacol. Sci. 1993 Jan; 14(1):13-20).

On the other hand, there are few studies on glutamic acid receptors other than in central nervous system. Glutamic acid is known to serve also as an energy source and a source for trapping ammonia that is no longer necessary and always exist in blood plasma in an amount on the order of several tens micromoles or more. In the central nervous system, the concentration of intracellular glutamic acid is on the order of nanomoles or less due to the existence of blood-brain barrier. As a result, the affinity of the above-mentioned glutamic acid receptor discovered in the central nervous system for glutamic acid is on the order of nanomoles to micromoles, so that the glutamic acid receptor can act only when glutamic acid is released from the nerve ending. In addition, the glutamic acid receptor tends to be inactivated or cause tachyphylaxis; in the central nervous system, gliacytes always take up glutamic acid through specific transporters to lower extracellular concentration of glutamic acid. On the other hand, in those sites other than the central nervous system where no protection by the blood-brain barrier is available, if glutamic acid receptors are expressed, it is considered that the glutamic acid receptors are always in a stimulated state and thus actually are inactivated and do not function.

However, in 2001, Chaudhari, N., Landin, A.M., Roper, S.D. et al. discovered low affinity glutamic acid receptor as an "umami" receptor from rat gustatory bud cells (Nat. Neurosci. 2000, Feb; 3(2):113-9). The umami receptor is gustatory type mGluR4, which has the same host gene as

type 4 (mGluR4), a subtype of rat brain type metabotropic type glutamic acid receptor (Tanabe, Y. et al., Neuron, 1992, Jan;8)1):169-79; Flor, P.J. et al., Neuropharamacology, 1995, Feb;34(2):149-55) and is partially defective of extracellular domain of the brain type mGluR4 due to splicing mutation.

Today, we have several pieces of knowledge that suggest physiological functions of the peripheral glutamic acid receptor (Berk, M., Plein, H., Ferreira, D., Clin. Neuropharmacol., 2001, May-Jun;24(129-32; Karim, F., J. Neurosci. 2001, Jun 1;21(11):3771-9; Berk, M., Plein, H., Belsham, B., Life Sci. 2000;66(25):2427-32; Carlton, S.M., Goggeshall, R.E., Brain Res. 1999, Feb 27;820(1-2):63-70; Haxhij. M.A., Erokwu, B., Dreshaj, I.A., J. Auton. Nerv. Syst. 1997, Dec 11;67(3):192-9; Inagaki, N., FASEB J. 1995, May;9(8):686-91; Erdo, S.L., Trends Pharamcol. Sci., 1991, Nov;12(11):426-9; Aas, P., Tanso, R., Fonnum, F., Eur. J. Pharamacol. 1989, May 2;164(1):93-102; Said, S.I., Dey, R.D., Dickman, K., Trends Pharmacol. Sci. 2001, Jul;22(7):344-5; Skerry, T.M., Genever, P.G., Trends Pharamcol., Sci. 2001, Apr;22(4):174-81).

Incidentally, for mammals including humans to make normal growth and maintain normal (healthy) life, it is necessary to orally take up necessary amounts of nutrients at necessary timing and excrete unnecessary matter. This is actually done by a digestive tract, which is a single tube consisting of oral cavity, stomach, small intestine and large intestine. The process of digestion and absorption is controlled by intrinsic intestinal neuroplexus and extrinsic cranial nerves. The judgment at

to whether or not to take a necessary nutrient is performed by integration in the brain of a pathway that the individual is aware of (taste) with an autonomous pathway that the individual is unaware of (visceral sense). It is considered that salty taste (sodium, potassium, etc.) serves as a marker of minerals and is useful for maintaining the osmotic pressure of the body fluid; the sweetness (glucose) serves as a marker of carbohydrates and is useful for supplementing energy; umami (sodium glutamate) serves as a marker of protein source and is useful for supplementing energy and body protein; and bitterness serves as a marker of toxic substances. That is, necessary nutrients are taken up relying on the tastes thereof. Then, if the necessary amounts are fully taken up is judged based on satiety that is obtained through a series of intracerebral processes of signal input to nucleus solitary tract from the afferent pathway of activated vagus nerve through nutrient sensors existing in the stomach, small intestine, and liver-portal vein (Bray, G.A., Proc. Nutr. Soc., 2000;59:373-84; Bray G.A., Med. Clin. North. Am. 1989:73:29). In addition, it is considered that digestion and absorption (secretion of digestive enzymes, enterokinesis, etc.) are controlled through efferent pathway of the vagus nerve. However, details of the mechanism are unclear. Then, finally satiety is obtained and eating behavior is terminated. In case of eating a substance toxic to the organism (poison), it is considered to be discharged by vomiting or diarrhea through humoral and neural responses. In this case too, details of the mechanism are unclear.

On the other hand, physiological studies on the mechanism of chemical sense in the digestive tract have been performed since a long time ago and it is supposed that there is a sensor that senses the content in the digestive tract (for the details, reference is made to Mei, N., J. Auton. Nerv. Syst., 1983;9:199-206; Mei, N., Lucchini, S., J. Auton, Nerv. Syst., 1992;41:15-8). The digestive sensors include a glucose sensor (Mei, N., J. Physiol. (Lond.) 1978, 282, 485-5-6), a temperature sensor (El Ouazzani, T., Mei, N., Exp. Brain Res. 1979;15;34:419-34), an osmotic pressure sensor (Mei, N., Garnier, L., J. Auton. Nerv. Syst., 1986;16:159-70), a pH sensor, an amino acid sensor (Mei, N., Physiol. Rev., 1985;65:211-37), and a pressure sensor (Barber, W.D., Burks, T.F., Gastroenterol Clin. North. Am. 1987; 16:521-4).

In particular, the sensor that recognizes glutamic acid was suggested by Niijima et al. from neural excitation that occurred when glutamic acid was administered in the digestive tract by using the technique of electrically grasping the neural activity in the stomach branch and abdominal cavity branch of the vagus nerve that controls mainly the stomach and small intestine, assuming that there is a mechanism that recognizes glutamic acid in the vagus nerve ending (Niijima, A., Physiol. Behav., 1991;49:1025-8).

Disclosure of the Invention

Although many studies have been made on the glutamic acid receptor and digestive tract sensors as described above, to date, the substance of the glutamic acid sensor

is unclear and no progress has been made in the studies. Failure of isolation of a receptor mechanism containing a glutamic acid sensor (receptor, transporter, etc.) that is necessary for the recognition of nutrients on the mucous membrane of the digestive tract prevented progress of research in this field. The inventors of the present invention thought that elucidation of the substance of glutamic acid sensor in the digestive tract would enable development of drugs and the like directed to control of the nutrient recognition mechanism described below.

That is, the nutrient recognition mechanism also plays an important role on satiety or surfeit and improves poor physical condition due to edacity and imbalance of taken-up nutrients due to deviated food habit. It is considered that anomaly in the recognition of nutrients in the digestive tract naturally results in disturbance in the overall process of digestion and absorption, thus causing edacity, deviated food habit, inappetence, indigestion, diarrhea, constipation, etc. More medically, it is considered to be the factor for digestive ulcers (stomach ulcer, duodendum ulcer) due to psychogenetic hyperphagia, cibophobia, obesity, anomaly of acid secretion, anomaly of blood flow in digestive tract, anomaly of secretion of digestive enzymes, etc., stress ulcers, drug-caused (NSAIDs, etc.) acute ulcers, ischemic ulcer (ischemic colitis), diabetes due to anomaly of secretion of insulin or anomaly of secretion of digestive tract hormone, heavy stomach, nausea, constipation, diarrhea, hypersensitivity vowel syndrome, etc. due to anomaly of motility and so forth.

Further, in recent years, abrupt increase in the obesity rate is a social phenomenon, being a social issue. Many of those who are obese are said to have decreased basal metabolism and tend to eat too much. How to control the appetite of those obese is of a great social concern. Many try to be on an undue diet. However, in most cases, they are unsuccessful. Thus, improving the mechanism of recognizing nutrient in the digestive tract and obtaining satiety by meal normally is very important to those who are obese.

The present invention has been made from the above-mentioned viewpoint and it is an object of the present invention to elucidate the substance of the glutamic acid sensor in the digestive tract and provide a technology utilizing it.

The inventors of the present invention have studied the distribution of receptors in the digestive tract by an immunohistological technique using an antibody that recognizes intracellular domain of metabotropic glutamic acid receptor (type 4). As a result, they have found that in the mucous membrane layers of small intestine and large intestine, there are type 4 metabotropic glutamic acid receptor (GluR4)-positive cells. In the small intestine and large intestine, mucus-producing cell (goblet cell) was mGluR4-positive. Then, cDNA that is supposed to be a novel glutamic acid receptor was successfully cloned from the samples of the small intestine and large intestine. It is highly probable that the glutamic acid receptor is a glutamic acid sensor in the digestive tract and it is considered that the cDNA of the receptor, purified

receptor and cell expressing the receptor are useful for screening agents for controlling the function of the glutamic acid sensor in the digestive tract.

The present invention has been accomplished based on the above-mentioned findings and the gist of the present invention is as follows.

(1) A glutamic acid receptor protein having the following properties:

(A) it has a transmembrane domain and an intracellular domain common to those of type 4 metabotropic glutamic acid receptor proteins; and

(B) it has an extracellular domain by about 316 or 327 amino acid residues shorter than that of the type 4 metabotropic glutamic acid receptor protein.

(2) The glutamic acid receptor protein according to (1), wherein the protein is expressed in rat small intestine and large intestine.

(3) The glutamic acid receptor protein according to (1), wherein the protein comprises an amino acid sequence consisting of an amino acid sequence shown in SEQ ID NO: 7 or an amino acid sequence consisting of amino acids numbers 12 to 584 in the amino acid sequence shown in SEQ ID NO:7.

(4) The glutamic acid receptor protein according to (3), wherein the sequence includes substitution, deletion, insertion or addition of one or a plurality of amino acid residues, and the protein can generate a second messenger by binding glutamic acid thereto.

(5) A DNA which encodes a glutamic acid receptor protein according to any one of (1) to (4) and does not express

type 4 metabotropic glutamic acid receptor protein.

(6) A cell harboring a DNA which encodes the glutamic acid receptor protein according to any one of (1) to (4) in an expressible form.

(7) A method of producing glutamic acid receptor protein, comprising the steps of cultivating a cell harboring a DNA which encodes the glutamic acid receptor protein according to any one of (1) to (4) in an expressible form in a medium to produce the glutamic acid receptor protein, and collecting the glutamic acid receptor protein from the cell.

(8) A method of screening an agonist, an antagonist, or an allosteric modulator of glutamic acid, comprising the steps of reacting the glutamic acid receptor protein according to any one of (1) to (4) with a substance that binds to the protein in the presence of a test substance and detecting inhibition or promotion of the reaction.

(9) A method of screening an agonist of glutamic acid, comprising the steps of reacting a glutamic acid receptor protein according to any one of (1) to (4) and a test substance, and detecting the reaction.

(10) The method according to (8), wherein the cell according to claim 6 or a membrane fraction prepared from the cell is used as the glutamic acid receptor protein.

(11) The method according to (10), in which the inhibition or promotion of the binding is detected by a second messenger generated by the glutamic acid receptor protein.

(12) The method according to (9), wherein the cell according to claim 6 or a membrane fraction prepared from the cell is used as the glutamic acid receptor protein.

(13) The method according to (12), in which the inhibition or promotion of the binding is detected by a second messenger generated by the glutamic acid receptor protein.

(14) An antibody that specifically binds to the glutamic acid receptor protein according to any one of (1) to (4).

(15) A method of producing a drug for modulating a second messenger generated by binding glutamic acid to a glutamic acid receptor, comprising the steps of:

reacting the glutamic acid receptor protein according to any one of (1) to (4) with a substance that binds to the protein in the presence of a test substance and detecting inhibition or promotion of the reaction to screen an agonist, an antagonist, or an allosteric modulator of glutamic acid; and

preparing a pharmaceutical composition containing the agonist, antagonist, or allosteric modulator of glutamic acid obtained in the reacting step as an active ingredient.

(16) A method of producing a drug for modulating a second messenger generated by binding glutamic acid to a glutamic acid receptor, comprising the steps of:

reacting the glutamic acid receptor protein according to any one of (1) to (4) with a test substance and detecting the reaction to screen an agonist of glutamic acid; and

preparing a pharmaceutical composition containing the agonist of glutamic acid obtained in the reacting step as an active ingredient.

Hereinafter, the present invention will be described in detail.

The glutamic acid receptor protein of the present invention typically has an amino acid sequence consisting of amino acids numbers 12 to 584 in the amino acid sequence described in SEQ ID NO: 7 in the Sequence Listing. The open reading frame of the nucleotide sequence of rat cDNA encoding the protein is shown in SEQ ID NO: 6. In the 5'-terminal region of this sequence, there were found two methionine codons (base numbers 1 to 3, and 34 to 36) each of which is possibly the start codon. Although it is highly possible that the methionine codon on the N-terminal side is the start codon, it is also possible that the second methionine codon is the start codon. In any event, ligation of a suitable promoter to upstream of the nucleotide sequence shown in SEQ ID NO: 6 and expression of the resultant in a suitable cell enables production of an active glutamic acid receptor.

Comparison of the amino acid sequence described in SEQ ID NO: 7 with the brain type metabotropic glutamic acid type 4 receptor (mGluR4) indicated that the c-terminal sides (amino acids numbers 15 to 584 in SEQ ID NO: 7) matched but the N-terminal side of the amino acid sequence described in SEQ ID NO: 7 was shorter by 300 amino acid residues or more than mGluR4. As described later, the glutamic acid receptor protein of the present invention was considered to be a splicing variant derived from the gene common with that of mGluR4. Hereinafter, the glutamic acid receptor protein of the present invention will also be referred to as mGluR4 variant in some cases.

Fig. 1 shows the structures of mGluR4 and mGluR4 variant. mGluR4 consists of an intracellular domain, 7

transmembrane domains and an extracellular domain. The mGluR4 variant also has an intracellular domain and 7 transmembrane domains that have the same sequences as those of mGluR4. On the other hand, the extracellular domain of the mGluR4 variant is shorter by 316 amino acid residues if the first methionine codon is a start codon, or by 327 amino acids if the second methionine codon is a start codon, than mGluR4.

That is, the mGluR4 variant of the present invention has common transmembrane domain and intracellular domain with those of the type 4 metabotropic glutamic acid receptor protein and an extracellular domain that is by about 316 or 327 amino acid residues shorter than the type 4 metabotropic glutamic acid receptor protein.

Comparison of the cDNA sequence encoding the above-mentioned mGluR4 variant with the mRNA sequence of mGluR4 (O'Hara, et al., Neuron, 11:41, 1993) suggested that these are derived from the common gene. That is, generation of mGluR4 variant is presumed to be a result of elimination of the second exon in the mGluR4 gene by alternative splicing to generate frame shift, which in turn causes a stop codon to appear in the first exon and causes a start codon downstream thereof.

The mGluR4 variant of the present invention may be derived from a rat. Alternatively, so long as it can generate a second messenger when glutamic acid is bound thereto, the mGluR4 variant may be derived from any animal including mammalian such as human, monkey, mouse, dog, cow, and rabbit, birds, and fin. In the case where the mGluR4 variant is used as a component of pharmaceutical

composition, it is preferably derived from a mammalian.

The mGluR4 variant of the present invention may be a protein having the amino acid sequence of SEQ ID NO: 7 or the amino acid sequence consisting of amino acid numbers 12 to 584 in SEQ ID NO: 7, or a protein having the amino acid sequence of SEQ ID NO: 7 including substitution, deletion, insertion or addition of one or a plurality of amino acids at one or a plurality of sites so long as it has properties that it can generate a second messenger when glutamic acid is bonded thereto.

The "plurality" as used herein varies depending on the positions of amino acid residues in the three-dimensional structure of the protein and the types of the amino acids, however, the number may be such that the homology with the amino acid sequence shown by SEQ ID NO: 7 is 80% or more, preferably 90% or more. More particularly, the plurality is 2 to 115, preferably 2 to 58, more preferably 2 to 30.

The glutamic acid receptor of the present invention may be in a purified or isolated form; however, when the activity is required, it is preferably in a form that is expressed in a suitable cell and localized in the membrane of the cell or in a form contained in a membrane fraction prepared from a cell in which the mGluR4 variant was expressed. Thus, the glutamic acid receptor of the present invention also includes cells that express mGluR4 variant or a membrane fraction prepared from such cells.

The mGluR4 variant can be obtained, for example, by introducing DNA that encodes the mGluR4 variant into a suitable host cell to express the mGluR4 variant. The

above-mentioned DNA includes gene that encodes the mGluR4 variant, isolated from the chromosome of a cell of a mammalian such as mouse. When chromosomal gene is used, it is preferable that cDNA is used since it is considered necessary to control a post-transcriptional process such as splicing so that mGluR4 variant can be generated.

The cDNA of mGluR4 variant can be cloned by amplifying the cDNA of mGluR4 variant using RNA prepared from the small intestine or large intestine of a mammal such as a rat as a template and oligonucleotides shown in, for example, SEQ ID NOS: 1 to 5 as primers. In addition, since the structure of mGluR4 variant, particularly unique structure on the N-terminal region has been made clear by the present invention, cloning and identification of the cDNA of mGluR4 variant can be performed easily based on the structures. The open reading frame nucleotide sequence of the thus obtained cDNA of mGluR4 variant is shown in SEQ ID NO: 6.

The DNA which encodes the mGluR4 variant includes in addition to the nucleotide sequence shown in SEQ ID NO: 6, DNA which hybridizes with DNA having this nucleotide sequence or a probe that can be prepared from the same nucleotide sequence under stringent conditions and that encodes the mGluR4 variant. The "stringent conditions" means conditions whereby specific hybrid is formed but nonspecific hybrids are not formed. It is difficult to clearly express the conditions by numeric values; examples thereof include those conditions whereby DNAs having high homology, for example, DNAs having 50% or more, preferably 75% or more homology hybridize with each other but DNAs

having a lower homology than that will not hybridize with each other, or those conditions whereby DNAs hybridize with each other under ordinary washing conditions in southern hybridization, i.e., at 60°C and a salt concentration corresponding to 1 × SSC, 0.1% SDS, preferably 0.1 × SSC, 0.1% SDS.

Cells to which DNA encoding the mGluR4 variant is introduced include preferably animal cells, insect cells or yeast when the activity of mGluR4 variant is necessary, with animal cells being particularly preferable. Examples of cells that are considered to enable transient expression of the function by introducing a recombinant vector containing DNA encoding the mGluR4 variant include *Xenopus laevis* oocyte, Chinese hamster ovary (CHO), baby hamster kidney (BHK) cell, human embryonic kidney (HEK) cell, Sf-9 insect cell, PC12 cell, and COCA-2 cell. In addition, when DNA encoding the mGluR4 variant is incorporated in chromosomal DNA to express the mGluR4 variant permanently, those cells other than the *Xenopus laevis* oocyte are suitable.

On the other hand, when no physiological activity is necessary such as the case where the mGluR4 variant is used as an immunogen for preparing antibody that specifically binds to the mGluR4 variant, cells to which DNA encoding the mGluR4 variant is introduced may be those cells that do not express the mGluR4 variant in an active form. As such cells, microbial cells that are usually used for the production of heterologous protein, including *Escherichia coli* may be used.

To produce the mGluR4 variant in the host cell, DNA,

which encodes the mGluR4 variant, is ligated to an expression regulation sequence such as promoter or enhancer suitable for the host cell. The DNA which encodes the mGluR4 variant may include a processing information site, for example, a ribosome binding site, an RNA splicing site, a polyadenylation site, and a transcription terminator sequence as necessary. Preferable expression control sequences include promoters derived from immunoglobulin gene, SV40, adenovirus, bovine papilloma virus, and cytomegalovirus.

The techniques necessary for the manipulation of cells such as introduction of DNA therein are described in, for example, Sambrook, J., Fritsch, E.F., and Maniatis, T., "Molecular Cloning A Laboratory Manual, Second Edition", Cold Spring Harbor Laboratory Press, (1989).

The mGluR4 variant and a cell that retains the mGluR4 variant can be produced by cultivating a cell that harbors the DNA encoding the mGluR4 variant obtained as described above in an expressible form in a medium to produce the mGluR4 variant.

Active mGluR4 variant, that is, mGluR4 variant that can generate a second messenger when glutamic acid is bound thereto can be utilized for screening agonist, antagonist or allosteric modulator of glutamic acid. For example, the mGluR4 variant and a substance that binds to the mGluR4 variant are reacted in the presence of a test substance, and inhibition or promotion of the reaction is detected, thereby screening agonist, antagonist or allosteric modulator of glutamic acid (hereinafter, these may be referred to collectively as "ligand"). The

allosteric modulator binds to a site other than the binding site between the mGluR4 variant and glutamic acid to exhibit similar function to that of the agonist or antagonist.

Further, the agonist of glutamic acid may be screened by reacting the mGluR4 variant with a test substance and detecting the reaction.

The active mGluR4 variant may include cells that express the mGluR4 variant or membrane fractions prepared from such cells. Such membrane fractions may be prepared by allowing cells to express active mGluR4 variant, ultrasonically disrupting the cells, and subjecting the sonicate to density gradient centrifugation to collect a membrane fraction.

Further, examples of the substance that binds to the above-mentioned mGluR4 variant include glutamic acid, glutamic acid agonist, or known ligands that bind to mGluR4 (L-AP4, CPPG, MAP-4, or the like). The substances that modulate the activity of the mGluR4 variant include drugs that influence the intracellular concentration of calcium (calcium channel and sodium channel opener, Na/K pump inhibitor, Na/Ca exchange agonist, Ca-ATPase inhibitor, protein kinase C agonist), drugs that influence intracellular cAMP concentration (phosphodiesterase agonist, adenylate cyclase agonist), and drugs that influence intracellular cGMP concentration (cGMP-dependent phosphodiesterase agonist, guanylate cyclase agonist) and so forth.

Inhibition or promotion of the reaction between mGluR4 variant and a substance that binds thereto can be

detected by measuring a second messenger that is generated by binding of a ligand such as glutamic acid to the mGluR4 variant. Alternatively, the above-mentioned inhibition or promotion of reaction can also be detected by measuring the binding of a labeled known ligand to the mGluR4 variant instead of detecting the second messenger.

Further, the reaction between the mGluR4 variant and the agonist of glutamic acid can be detected by detecting a second messenger that is generated by binding of the mGluR4 variant to the agonist of glutamic acid.

The intracellular domain of mGluR4 variant is the same as the brain type and gustatory bud type mGluR4 and the brain type and gustatory bud type mGluR4 have the same intracellular signal transmitting mechanism. Therefore, the mGluR4 variant is expected to have similar intracellular signal transmitting mechanism. Therefore, the above-mentioned second messenger is inhibition of production of intracellular cAMP accompanying activation of Gi (inhibitory GTP binding protein) to adenylate cyclase. Furthermore, downstream of cAMP in signal transmission includes regulation of gene expression through modulation of cAMP-responsive element and modulation of function of acute phase by phosphorylation of cytoplasm or membrane protein. Therefore, the second messenger can be detected by measuring change in the amount of cAMP accumulated in the cell, measuring change in intracellular calcium concentration through modulation of intracellular Ca-ATPase activity, or measuring change in the function of channel.

Hereinafter, examples of concrete method for

screening ligands by using the mGluR4 variant will be explained below.

(1)   The mGluR4 variant cRNA and CFTR (cystic fibrosis transmembrane conductance regulator) cRNA are allowed to coexpress in *Xenopus laevis* oocytes and screening of ligands that act on the mGluR4 variant is performed by a two electrode voltage clamp method by using increase or decrease in chloride current attributable to CFTR as an index (Uezono et al., Receptors Channels 1993;1(3):233-41; Cunningham SA et al., Am. J. Physiol 1992 Mar;262(3 Pt 1):C783-8). CFTR is a causative gene product for cystic lung fibrosis and is a chloride channel whose activity is modulated by intracellular cAMP.

(2)   The mGluR4 variant expressing cells and a candidate compound for ligand are allowed to coexist for a certain period of time, and then the amount of intracellular cAMP in the expressing cell is determined and screening of ligands is performed based on increase or decrease in the cAMP level (Chaudhari N, Nat Neurosci 2000 Feb;3(2):113-9; Flor PJ, Neuropharmacology 1995 Feb;34(2)149-55). The amount of cAMP can be measured by using commercially available kits.

(3)   Screening of ligands is performed by reacting a candidate compound for ligand and a known ligand that acts on mGluR4 (for example, glutamic acid, L-AP4, CPPG, MAP-4 or the like) on an mGluR4 variant expressing cell or a membrane fraction prepared from the cells for a certain period of time and measuring the amount of the known ligand bound to the cell membrane or membrane fraction of the mGluR4 variant expressing cells (Naples MA,

Neuropharmacology 2001;40(2):170-7; Thomsen C,
Neuropharmacology 1997 Jan;36(1)21-30; H.I. Yamamura, S.J.
Enna and M.J. Kuhar eds, 1958, Neurotransmitter Receptor
Binding, 2nd ed., Raven Press, New York). The amount of
the known ligand can be measured by labeling a portion of
the substances with radioactivity and determining the
quantity of radioactivity bound to the cell membrane or
membrane fraction.

(4) A calcium-sensitive dye (for example, Fura-2, Indo-1,
Fluo-3 or the like) is introduced into an mGluR4 variant
expressing cell in advance, and a ligand candidate
compound and the mGluR4 variant expressing cells are
allowed to contact for a certain period of time, and then
ligands are screened by using as an index a change in a
ratio of intensities of fluorescence (intracellular
calcium concentration). Alternatively, screening of ligand
is performed by a change in a ratio of intensities of
fluorescence (intercellular calcium concentration)
obtained when an mGluR4 variant agonist, a candidate
compound for ligand, and an mGluR4 variant expressing
cells into which a calcium-sensitive dye is introduced
are allowed to contact for a certain period of time.

(5) Screening of ligands is performed by using as an
index a change in a ratio of intensities of fluorescence
(intracellular cAMP concentration) obtained when a cAMP-
sensitive fluoroprotein (for example, FICRhR or the like)
is introduced into an mGluR4 variant expressing cell in
advance and then a ligand candidate compound and the
mGluR4 variant expressing cells are allowed to contact for
a certain period of time (Adams SR, Nature 1991 Feb

21;349(6311):694-7).

(6)   Screening of ligands is performed by using as an index the production amount of proton obtained when a candidate compound for ligand and an mGluR4 variant expressing cells are allowed to contact for a certain period of time, or when an mGluR4 variant agonist, a candidate compound for ligand and an mGluR4 variant expressing cells are allowed to contact for a certain period of time and measured by a cytosensor (McConnell HM, Science 1992 Sep 25;257(5078):1906-12).

A pharmaceutical composition that comprises the agonist, antagonist or allosteric modulator of glutamic acid as an active ingredient thus screened can be used as a drug for modulating the second messenger generated when glutamic acid is bound to a glutamic acid receptor. Modulation of the second messenger enables improvement and prevention of diseases and pathology caused by anomaly of glutamic acid receptor.

The anomalies of control of vagus nerve include anomaly of afferent pathway (disorder of nutrient recognition) and anomaly of efferent pathway. The diseases or pathology due to the anomaly of afferent pathway include hyperphagia, cibophobia, obesity and so on. On the other hand, those due to the anomaly of efferent pathway include digestive ulcers (stomach ulcer, duodendum ulcer) due to psychogenetic hyperphagia, cibophobia, obesity, anomaly of acid secretion, anomaly of blood flow in digestive tract, anomaly of secretion of digestive enzymes, etc., stress ulcers, drug-caused (NSAIDs, etc.) acute ulcers, ischemic ulcer (ischemic colitis), diabetes due to

anomaly of secretion of insulin or anomaly of secretion of

digestive tract hormone, heavy stomach, nausea,

constipation, diarrhea, hypersensitivity vowel syndrome,

etc. due to anomaly of motility and so forth.

Use of mGluR4 variant as an immunogen enables

preparation of an antibody that specifically binds to the

mGluR4 variant. In particular, since the mGluR4 variant

has a novel amino acid sequence in the N-terminus,

antibody, particularly monoclonal antibody, that contains

this portion as an epitope is expected to bind to the

mGluR4 variant and not to bind to other glutamic acid

receptors. The antibody specific to the mGluR4 variant can

be used in immunostaining specific to the mGluR4 variant.

Brief Description of the Drawwings

Fig. 1 is a schematic diagram showing the structures

of mGluR4 and mGluR4 variant.

Fig. 2 is a photograph showing results of

immunostaining with an anti-mGluR4 antibody. (A): small

intestine. (B): large intestine.

Fig. 3 is a diagram showing action of L-glutamic

acid on afferent action of the abdominal branch of vagus

nerve, with horizontal axis indicating time and vertical

axis showing neuronal action.

Best Mode for Carrying out the Invention

Hereinafter, the present invention will be described

in detail through examples.

Example 1: Cloning of novel metabotropic glutamic acid

receptor cDNA from rat intestinal tissue

Reverse transcription was performed by using superscript (Gibco-BRL) and SMART (Switching Mechanism at 5' end of RNA Transcript) RACE (rapid amplification of cDNA ends) cDNA amplification kit (Clontech) and using total RNA derived from small intestine and large intestine of a Wistar rat (CRJ) as a template. By using the obtained cDNA as a template, the 5' end fragment of rat mGluR4 was amplified by 5'-RACE using LA taq (TaKaRa) followed by nested PCR.

Gene specific primers (R-mGluR4: 5'-GAA GTT GAC GTT CCT GAT GTA CT-3' (SEQ ID NO: 1), R2-mGluR4: 5'-ACA GCG TCA ATC ACG AAC TGC AC-3' (SEQ ID NO: 2)) were synthesized based on the brain type mRNA sequence (O'Hara, et al., Neuron, 11:41, 1993).

The primers from SMART-RACE kit (Universal primer mix: Long 5'-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA ACA ACG CAG AGT-3' (SEQ ID NO: 3), Short 5'-CTA ATA CGA CTC ACT ATA GGG C-3' (SEQ ID NO: 4), and Nested universal primer: 5'-AAG CAG TGG TAA CAA CGC AGA GT-3' (SEQ ID NO: 5)) were used for performing 5'-RACE PCR.

About 30 ng rat cDNA was amplified in 10 μl 10×LA PCR buffer, 2.5 mM MgCl$_2$, 2.5 mM dNTP mixure and 0.25 units La Taq enzyme by using gene-specific primer, R-mGluR4 and Universal primer mix.

PCR was performed 40 cycles by using a GeneAmp PCR system 9700 at 94°C for 20 seconds, 50°C for 1 minute, and 68°C for 3 minutes, followed by chain extension at 68°C for 10 minutes.

The obtained reaction product was amplified again by using R2-mGluR4 and Nested universal primer under the same

conditions as described above to obtain a 300 bp fragment.

The amplification product was separated by electrophoresis on agarose and cloned in pCR-II vector having dual promoter by using TA cloning kit (Invitrogen).

Analysis of the nucleotide sequence of the above-mentioned fragment by using ABI Sequencer Model 3700 (ABI) identified clones named 010411-70, 010411-66, and 010528-3 from the large intestine and 010528-37 from the small intestine. They contained the same nucleotide sequence.

The nucleotide sequence identified from each of the above-mentioned clones is shown in SEQ ID NO: 6. In addition, the amino acid sequence encoded by the open reading frame contained in the nucleotide sequence is shown in SEQ ID NO: 7. Comparison of the nucleotide sequence with the mRNA sequence of brain mGluR4 (O'Hara, et al., Neuron, 11:41, 1993) revealed that in the nucleotide sequence of SEQ ID NO: 6, cytosine at position 1724 is directly liked to adenine at position 1880 (the positions of nucleotides are based on mGluR4 mRNA) and that 155 bp fragment is deleted. This suggests that the frame shift in the domain upstream of cytosine at position 1724 results in translation of a novel peptide (SEQ ID NO: 8) having methionine at the N-terminus that is encoded by a start codon starting at 1684 or 1717 and that the peptide linkes to glycine at position 343 located in the extracellular domain of the glutamic acid receptor. The remaining sequence was the same as the sequence of brain type receptor. A codon that starts at position 1684 is shown as the start codon in SEQ ID NO: 6 and SEQ ID NO: 7. Although it is unclear which of the two methionine codons

the start codon is, it is expected in any event that introduction of the open reading frame and its peripheral sequence and expression thereof in a cell can give rise to the same expression product as that expressed in living organism.

The above-mentioned protein encoded by the open reading frame has a molecular weight of about 88 kD assuming that the first methionine in the amino acid sequence shown in SEQ ID NO: 7 is the N-terminus. Fourteen amino acids on the N-terminus does not exist in the amino acid sequence encoded by mGluR4 mRNA.

From the above, it is demonstrated that the obtained clone is a splicing variant of mGluR4 having a novel extracellular domain.

Example 2: Identification of localization of glutamic acid receptor by immunostaining
<1> Preparation of sliced samples of rat small intestine and large intestine

Rats (Wistar strain, male, 10 to 15-week age) were let bleed by dissecting right auricle of the heart under anesthesia with ether and immediately thereafter, small intestine and large intestine were extracted. From the small intestine, a part about 5 cm from the outlet of stomach was extracted. From the large intestine, about 7 cm long part from the ileocecal opening on the side of rectum was extracted. When a large amount of digestion product remained in the intestine, the intestinal tract was washed with physiological saline.

The dissected intestinal tract was cut open, put on

a cork board with a needle, and immersed in 4%
paraformaldehyde (4°C) with shaking for one day to fix it.
Thereafter, the intestinal tract was immersed in 20%
Sucrose-PBS for 3 to 4 days for cryprotection, wrapped in
Tissue-Tek[R] (OCT compound), and then sliced to a thickness
of 5 to 7 μm in a cryostat. The sections were dried at
room temperature and then stored at 4°C until use for
various kinds of staining.

<2> Immunostaining wit anti-metabotropic glutamic acid
receptor antibody

Immunostaining of the sections was performed
according to the method described in Drengk, A.C. et al.,
J. Auto. Nerv. Sys. 78: 109-112, 2000, and Miampamba, M.
et al., J. Auto. Nerv. Sys. 77: 140-151, 1999. The
sections were first washed with PBS and then treated with
3% hydrogen peroxide/methanol for 15 minutes in order to
inhibit a reaction by endogenous peroxidase. Then, the
sections were washed with PBS and blocked with 1% bovine
serum albumin added PBS (1% BSA-PBS) containing 10% normal
equine serum for 1 hour. After washing with PBS again, the
sections were reacted with a primary antibody diluted with
1% BSA-PBS containing 1% normal equine serum (Table 1) at
4°C for two overnights. Thereafter, the sections were
washed with PBS and reacted with secondary antibody
diluted with 1% BSA-PBS (Table 1) at room temperature for
1 hour. Finally, ABC (Avidin-Biotin Complex) reaction was
performed using a Vectorstain elite kit (Vector), followed
by color development with 0.025% diaminobenzidine-0.25%
nickel chloride-0.01% H_2O_2. After completion of the
reaction, the sections were washed with PBS, dehydrated

with ethanol/xylene, and sealed and then observed under a
microscope. A sample without primary antibody was used as
a negative control. The kinds and dilution magnifications
of primary antibody and of secondary antibody are shown in
Table 1.

Table 1

Primary antibody	Magnifications of primary antibody	Secondary antibody	Magnifications of primary antibody
anti-mGluR1a, rabbit, polyclonal, Chemicon, cat# AB1551	100	Biotinylated anti-rabbit Ig, Amersham Pharmacia Biotech, cat# RPN 1004	150
anti-mGluR2/3, rabbit, polyclonal. Chemicon, cat# AB1553	100	Biotinylated anti-rabbit Ig, Amersham Pharmacia Biotech, cat# RPN 1004	150
anti-mGluR4a, rabbit, Upstate Biotechnology, cat# 06-765	400	Biotinylated anti-rabbit Ig, Amersham Pharmacia Biotech, cat# RPN 1004	150
anti-mGluR5, rabbit, polyclonal, Chemicon, cat# AB5323	400	Biotinylated anti-rabbit Ig, Amersham Pharmacia Biotech, cat# RPN 1004	150

<3> Alcian Blue/PAS stain

The sections were washed with running water, treated
with 3% acetic acid, and then reacted with Alcian Blue
solution (pH 2.5, WAKO) for 30 to 40 minutes. Then, the
sections were washed with running water and immersed in 1%
periodic acid solution for 10 minutes, followed by

reaction with Schiff's reagent (WAKO) for 8 to 10 minutes. By the above treatment, mucin was stained to identify goblet cell of the intestinal tract. After washing the sections with running water again, finally, the sections were subjected to nuclear stain with Mayer's hematoxylin (WAKO), and after thus performing color intensification, the sections were dehydrated and sealed.

<4> Results

The results of immunostaining are shown in Fig. 2. Regarding the small intestine (Fig. 2A) and large intestine (Fig. 2B), the goblet cells were stained with the anti-mGluR4. It is a general assumption that mGluR4 receptors are not expressed in the goblet cells. Therefore, it is conceived that the mGluR4 variant is expressed in the goblet cells, which suggests that the mGluR4 variant is functionally related to secretion of mucoid.

Example 3: Presumption of the function of novel mGluR4 variant

After starved for 18 hours, rats (Wistar strain, male, 8 to 10 week age: Japan Charles River) were laparotomized under anesthesis with urethane (1g/kg, i.p.) and about 5 mm of the abdominal cavity branch of vagus nerve was peeled off under stereoscopic microscope. After the cutting of vagus nerve fasciculus, the vagus nerve fasciculus was placed on a small operation stage (8×6 mm) and fat and connective tissue surrounding the vagus nerve fasciculus were carefully peeled off. The terminal fiber on the organ side was placed on a platinum-made bipolar electrode and insulated from the surrounding tissue with

liquid paraffin/vaseline (1:1) mixed solution. As an administration route of MSG (sodium L-glutamate, manufactured by Ajinomoto Corporation), a silicone tube was placed in the stomach and the origin part of duodendum orally.

The neural activity potential was amplified 10,000 folds by a micro potential amplifier (DAM-80 manufactured by WPI) and subjected to A/D conversion (Powerlab 4sp, manufactured by ADI Instruments) after noise was decreased by a Bessel filter (4-pole, High Cut 10 Hz, Low Cut 1 KHz). Thereafter, the potential was incorporated in a computer (sampling rate 3 KHz, iBook). Simultaneously, while monitoring the amplified signals by an oscilloscope, a noise component is separated from a neural signal component by a window discriminator (DSE-435, manufactured by DiaMedical Corporation), integrated for 5 seconds by a Spike Counter (DSE-335P, manufactured by Nihon Kohden Corporation) and recorded by a chart recorder (WT-465G, manufactured by Nihon Kohden Corporation). The waveform of spikes was analyzed by using SHE software (manufactured by ADI Instruments).

The results obtained are shown in Fig. 3. The afferent activity of the abdominal cavity branch of vagus nerve when 150 mM MSD was administered in the duodendum was increased. The afferent pathway of vagus nerve is considered to be a signal transmitting pathway that transmits visceral sense, particularly nutrient information from the stomach and intestines to the nucleus solitary tract and performs control of postcibal sense such as satiety or sense of displeasure and digestion by

modulating the efferent pathway of the vagus nerve. Therefore, increase in the activity of the afferent pathway of vagus nerve by the administration of MSG in the digestive tract indicates the possibility that MSG is a causative factor that generates the signal and the mGluR4 variant expressed in the lumen of the digestive tract mediates the generation of the signal.

Example 4: Preparation of novel mGluR4 variant expressing cell

<1> Preparation of novel mGluR4 variant expressing cell

A full-length cDNA of a novel mGluR4 variant was amplified again by a RT-PCR method by using PFU polymerase (Promega). Then, the resultant was cloned in pUni-V5-His-TOPO by using a cloning kit (Invitrogen) according to a conventional method and thereafter, the cDNA was incorporated into pcDNA3.1E by using a plasmid fusing method.

The prepared vector (pcDNA3.1E) was introduced in CHO-K1 cell (Dainippon Pharmaceutical Co., Ltd.) by using polyFect reagent (Qiagen). After 48 hours, neomycin (600 μg/ml) was added to a culture medium (Modified Eagle Medium (αMEM) containing 10% fetal bovine serum, 100 U/ml penicillin; Nacalai Tesque) and cultivation was continued for 2 to 3 weeks to perform screening of gene-introduced cells. The screened CHO-K1 cell was proliferated to a cell number that is necessary for the evaluation of function after expression of mRNA of novel mGluR1 variant was confirmed by an RT-PCR method.

<2> Measurement of function of mGluR4 variant gene by cAMP

measurement

.The variant expressing cells obtained as described above were dispensed to 2.5×10^5 cells/well on a plate (24-well) and cultivated for 20 hours and then used for the measurement of the function of cAMP. Each plate was washed well with Dulbeco's phosphate buffer and incubated for 20 minutes. Thereafter, stimulation was performed with each drug for 10 minutes at 37°C. After the stimulation, the cells were at once treated with ice-cooled 2.5% perchloric acid (PCA) for 30 minutes, neutralized with KOH, and centrifuged to obtain a supernatant (12,000 rpm × 10 min, 4°C). The cAMP content of the supernatant was determined according to a conventional enzyme immunoassay (ELISA) by using a cAMP assay kit (Amersham).

As a result, the amount of cAMP in the mGluR4 variant expressing cell was increased about 9 folds by treatment with a phosphodiesterase inhibitor (isobutyl-methyl-xanthine; IBMX; 100 μM) and an adenyl cyclase activator (forskolin; 10 μM). Comparison of the actions of glutamic acid (1 mM) and sodium chloride (1 mM) under the above conditions revealed that the amount of cAMP was 1,519 fmole in the sodium chloride added group (n=3) and 1,235 fmole in the glutamic acid added group (n=3). This made it clear that the amount of cAMP accumulated in the mGluR4 variant expressing cell was decreased by about 19% by the addition of glutamic acid. This experimental results demonstrated that the novel mGluR4 variant reacts with glutamic acid on the order of millimoles and modulates the signal transduction system.

Industrial Applicability

According to the present invention, a novel metabotropic glutamic acid receptor can be provided. The glutamic acid receptor can be used in screening agonists, antagonists or allosteric modulators of glutamic acid. In addition, the glutamic acid receptor can be used as a medicine for improving diseases and pathology due to metabolic disorder in digestive tracts such as small intestine and large intestine.